Expression of Epidermal Keratins and the Cornified Envelope Protein Involucrin is Influenced by Permeability Barrier Disruption

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In previous studies we have shown that experimental permeability barrier disruption leads to an increase in epidermal lipid and DNA synthesis. Here we investigate whether barrier disruption also influences keratins and cornified envelope proteins as major structural keratinocyte proteins. Cutaneous barrier disruption was achieved in hairless mouse skin by treatments with acetone ± occlusion, sodium dodecyl sulfate, or tape-stripping. As a chronic model for barrier disruption, we used essential fatty acid deficient mice. Epidermal keratins were determined by one- and two-dimensional gel electrophoresis, immunoblots, and anti-keratin antibodies in biopsy samples. In addition, the expression of the cornified envelope proteins loricrin and involucrin after barrier disruption was determined by specific antibodies in human skin. Acute as well as chronic barrier disruption resulted in the induction of the expression of keratins K6, K16, and K17. Occlusion after acute disruption led to a slight reduction of keratin K6 and K16 expression. Expression of basal keratins K5 and K14 was reduced after both methods of barrier disruption. Suprabasal keratin K10 expression was increased after acute barrier disruption and K1 as well as K10 expression was increased after chronic barrier disruption. Loricrin expression in mouse and in human skin was unchanged after barrier disruption. In contrast, involucrin expression, which was restricted to the granular and upper spinous layers in normal human skin, showed an extension to the lower spinous layers 24 h after acetone treatment. In summary, our results document that acute or chronic barrier disruption leads to expression of keratins K6, K16, and K17 and to a premature expression of involucrin. We suggest that the coordinated regulation of lipid, DNA, keratin, and involucrin synthesis is critical for epidermal permeability barrier function. ***Key word: loricrin. J Invest Dermatol 111:517–523, 1998***

The permeability barrier of the skin is localized in the stratum corneum and consists of protein-enriched corneocytes and lipid-enriched intercellular domains (Elias, 1983). The barrier is formed during terminal differentiation of the epidermis that results in corneocytes comprising an insoluble cornified envelope and cross-linked cytoskeletal proteins (Downing, 1992; Roop, 1995). The lipids for barrier function are synthesized in the keratinocytes of the nucleated epidermal layers, stored in the lamellar bodies, and extruded into the intercellular spaces during the transition from the stratum granulosum to the stratum corneum forming a system of continuous membrane bilayers (Elias, 1983; Menon *et al*, 1992). Several studies in hairless mouse skin have shown that barrier disruption leads to an increase in epidermal lipids synthesis including cholesterol, free fatty acids, and ceramides (Grubauer *et al*, 1987, 1989; Holleran *et al*, 1991). Also, we have shown that barrier disruption in acute [topical treatments by acetone, 10% sodium dodecyl sulfate (SDS), or by tape-stripping] and chronic models [essential fatty acid deficient diet (EFAD)] leads to an increase in DNA synthesis resulting in epidermal hyperproliferation (Proksch *et al*, 1991, 1992). Bulk protein synthesis is not increased after barrier disruption (Choi *et al*, 1992), but a variety of specific proteins, e.g. actin, LDL-Receptor, or enzyme proteins, are upregulated after acute barrier disruption (Jackson *et al*, 1992).

The purpose of our studies was to examine whether major structural proteins synthesized in the keratinocytes are influenced by permeability barrier function. The commonly used phenotypic markers of epithelial development and differentiation are keratins. They emanate from a perinuclear ring (free ribosomes), extend throughout the cytoplasm, and terminate at desmosomes and hemidesmosomes (Roop, 1995). During epidermal differentiation specific keratins are synthesized. Keratins 5 and 14 (K5 and K14) are expressed in basal keratinocytes, K1 and K10 in differentiating suprabasal keratinocytes, and K6 and K16 in suprabasal layers of psoriasis and in wound healing (Thewes *et al*, 1991; Wilson *et al*, 1992; Hagemann and Proksch, 1996; Paladini *et al*, 1996). K17 has been observed in suprabasal layers in interfollicular psoriatic skin (Jiang *et al*, 1993; Smack *et al*, 1994; McKay and Leigh, 1995; Fuchs, 1995; Leigh *et al*, 1995). Mutations in keratin genes can result in human diseases characterized by a disturbed epidermal homeostasis including a disturbed barrier function (Roop, 1995; Frost *et al*, 1968; Cheng *et al*, 1992; Chipev *et al*, 1992; Lane *et al*, 1992; Rothnagel *et al*, 1992).

The cornified envelope is a specialized structure that replaces the plasma membrane of terminally differentiating keratinocytes, it consists of proteins cross-linked by covalent bonds into a rigid scaffold, with

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Abbreviations: EFAD, essential fatty acid deficiency; TEWL, transepidermal water loss.

lipids (hydroxyceramides) covalently attached to its external surface and the filament-matrix complex (keratins) interacting with its internal surface (Ming *et al*, 1994; Roop, 1995; Steinert and Marekov, 1995, 1997). Involucrin is a cytoplasmatic protein synthesized by human squamous epithelial cells; it is expressed early in the keratinocyte differentiation process and is a major early isopeptide cross-linked component of the keratinocyte cornified cell envelope (Rice and Green, 1979; Kanitakis *et al*, 1987; Robinson *et al*, 1996; Steinert *et al*, 1997). Involucrin can be detected in the cell periphery of high stratum spinosum cells by using specific antibodies. In hyperproliferative diseases like psoriasis, premature expression of involucrin is found in the lower spinous layers (Kanitakis *et al*, 1987; Hohl, 1990; McKay *et al*, 1995).

Recently, Marekow *et al* provided evidence that covalently bound lipids are linked to involucrin.[1] Loricrin, also a cornified envelope protein, is expressed very late in epidermal differentiation in the granular layers of normal mouse and human epidermis. Loricrin initially accumulates in keratohyaline granules, termed L-granules. Subsequently, loricrin becomes cross-linked as a major component of the cornified envelope (Hohl, 1990; Steinert *et al*, 1995). A coordinated expression of loricrin with other epidermal proteins and the appearance of lipid lamellar granules in mouse gestational development has been reported (Bickenbach *et al*, 1995).

We examined in acute and chronic models whether artificial permeability barrier disruption leads to changes in epidermal proliferation- and differentiation-related keratins and to changes in the cornified envelope proteins loricrin and involucrin. We found pronounced changes in these structural keratinocyte proteins after permeability barrier disruption.

MATERIALS AND METHODS

Animals Female hairless mice [Crl:(hr/hr) BR] aged of 3 or 6–8 wk were supplied by Charles River (Sulzfeld, Germany). The animals were maintained conventionally under standardized conditions (room temperature, 22 ± 1°C; relative humidity, 55 ± 5%; 10 air changes per h; 12 h day/12 h night cycle) in groups in plastic cages with polyester filter covers on wood granulate bedding. The 3 wk old animals were fed either with standard laboratory animal chow or with an EFAD diet (Bavandi *et al*, 1992), modified by increasing $MgSO_47H_2O$ to 2.38 g per kg. Food and water was supplied ad lib.

Experimental protocol Acute disruption of the permeability barrier was induced by absolute acetone, by 10% SDS (in water) (treatments for 2–5 min with acetone- or SDS-soaked cotton swabs, respectively), or by tape-stripping (cellophane tape, Scotch type, six to eight times) until a 20–30-fold increase in transcutaneous/transepidermal water loss (TEWL) was achieved (Meeco electronic water analyzer, Meeco, Warrington, PA). Occlusion after barrier disruption was achieved by immediately inserting the mouse into a thumb of powderless latex gloves for 24 h as described previously (Grubauer *et al*, 1987). For each experiment at least three animals were treated and at least three animals served as control. At different points of time after treatment (3–72 h), skin samples of ~1 cm^2 from groups of 3–5 mice were obtained. Chronic barrier disruption in mice was achieved by maintenance with EFAD diet up to 18 wk. Skin samples were collected from three mice sacrificed after 3, 6, 9, 12, 15, and 18 wk on EFAD diet. Occlusion in EFAD mice was performed for 3 d in week 12. TEWL as a marker of barrier function was measured in these animals with an Evaporimeter (EP1, Servo Med, Kinna, Sweden). In human skin of three volunteers, barrier disruption was achieved by treatments with acetone-soaked cotton swabs for 15–30 min until a 4–6-fold increase in TEWL was achieved; at 12 h, 24 h, and 48 h, 4 mm punch biopsies were obtained. The study protocols were approved by the University of Kiel, by the Human Research Review Committee, and by the Committee of Animal Care, respectively.

Murine epidermal proliferation assay *in vivo* One hour before skin dissection 30 mg bromodesoxyuridine per kg (BrdU, # RPN 201, Amersham) were injected intraperitoneally. Five micrometer skin sections (prepared as described above) were processed by standard immunohistologic peroxidase technique using a monoclonal anti-BrdU-antibody (#RPN 201, Amersham) and diaminobenzidine as substrate as recommended by the supplier of the staining kit. The stained sections (one per mouse) were examined microscopically (160×) by counting the BrdU-labeled nuclei of interfollicular keratinocytes (cells in S-phase) in seven microscopic fields/sections. Labeling index in mice on an EFAD diet and in age-matched normally fed animals was defined by numbers of $BrdU^+$ cells per mm epidermal basal membrane. Morphometry was performed with an image analyzer (Videoplan, Kontron, Munich, Germany).

Determination of epidermal keratins and cornified envelope proteins

One-dimensional gel electrophoresis From full-thickness murine skin samples, the epidermis was removed by heat treatment (60°C, 3 min) and washed with 10 mM phosphate/147 mM saline/5 mM bis-(aminoethyl)-glycolether-N,N,N′,N′-tetraacetic acid (pH 7.4) and then homogenized (glass homogenizers) in 20 mM TRIS-HCI (pH 7.4), containing 1 Mm phenylmethylsulfonyl fluoride and 5 μg per ml each of pepstatin and antipain. The homogenate was extracted five times with 600 mM KCl/5 mM bis-(aminoethyl)-glycolether-N,N,N′,N′-tetraacetic acid/5 mM ethylene diamine N,N,N′,N′-tetraacetic acid disodium salt/1% Triton X-100/50 mM TRIS/Hcl [Tris-(hydroxymethyl)aminomethane] pH 7.4 and protease inhibitors as described (Wild and Mischke, 1986), with sonication (10 min) and subsequent centrifugation (5 min, 15,000×*g*). The resulting pellet was solubilized in sample buffer (2% SDS/5% mercaptoethanol/10% glycerol/0.001%bromophenol blue/62.5 Mm TRIS-Hcl pH 6.8) and heated to 95°C for 10 min as required for electrophoresis (Laemmli, 1970). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at limiting 600 V, 50 Ma, and 30 W with a Macrodrive power supply and a Multiphor II electrophoresis chamber for ≈1.5 h using 12.5% homogenous Excel Gels SDS and SDS buffer strips (all Pharmacia, Freiburg, Germany). Molecular weight markers from Novex (Mark 12) and Amersham (Rainbow Marker, Buckinghamshire, U.K.) were run in parallel. For Coomassie staining 20 μg, and for blotting/immunostaining 4 μg of the initial epidermis sample was applied to the gel. Fixing, Coomassie staining, destaining, and preserving of gels was performed as outlined by the manufacturer (Pharmacia). Dried gels were scanned and the images analyzed (not shown) and calculated (integrated) with a ImageMaster 1D Elite System (Pharmacia).

Two-dimensional gel electrophoresis For two-dimensional gel electrophoresis, epidermis and dermis were separated by heating to 60°C (3 min for murine skin, 5 min for human skin) in phosphate buffered saline. The epidermis was disrupted in a glass homogenizer with a high-salt buffer containing 1.5 M KCl, 10 mM NaCl, 0.5% Triton X-100, 2 mM dithioerythritol, and 10 mM Tris-HCl (pH 8.0), homogenized, and centrifuged at room temperature for 10 min (12,000 × *g*, four times). The final pellet was incubated with 20 μl GTC-buffer [4 M guanidinium thiocyanate, 0.1 mM Tris-HCl (pH 7.5), 1% mercaptoethanol] and homogenized at 65°C for 40 min. After centrifugation for 10 min (12,000 × *g*, 18°C) the supernatant was subjected to two-dimensional gel electrophoresis. The extracted keratins were analyzed by two-dimensional gel electrophoresis, using equilibrium isoelectric focusing or nonequilibrium pH gradient gel electrophoresis in the first dimension and SDS-PAGE with 9% acrylamide in the second dimension (O'Farell *et al*, 1977). Gels were stained with Coomassie brilliant blue R-250. The keratins were identified by comparing the results with published data (Moll, 1993; Schweizer, 1993; Zouboulis *et al*, 1993; Yoshikawa *et al*, 1995) and by immunoblotting using specific antibodies.

Immunostaining of keratin 6, involucrin, and loricrin in skin sections Murine skin samples were fixed in formalin and embedded in paraffin. After deparaffination and rehydration, 5 μm thick sections were incubated with 3% H_2O_2 for 5 min to block endogenous peroxidase activity and the rinsed sections exposed to 5 × 5 min microwave irradiation (650 W) for antigen detection according to Hazelbag *et al* (1995). After blocking nonspecific antibody binding by incubation with 20% normal pig serum (DAKO A/S, Denmark, 20 min, room temperature), the primary antibodies [anti-K6, 1:800, anti-loricin 1:500 or anti-involucrin (undiluted, Biomedical Technologies, Stoughton, MA; German distributor: Paesel & Lorei, Frankfurt, Germany)] were applied for 30 min at room temperature (Rosenthal *et al*, 1992; Hohl *et al*, 1993). A strep AB complex/HRP (DAKO A/S, 1:100, 30 min, room temperature) was used as a third antibody, followed by incubation with diaminobenzidine as peroxidase substrate.

Immunoblot analysis Two methods were used for one- and two-dimensional gel electrophoresis, respectively. (i) After blocking of the nonspecific binding sites with 3% gelatine in Tris-buffered saline the following staining protocol was used: as primary antibody, the monoclonal mouse anti-keratin antibodies CK 8.12 (Biomakor, Rehovot, Israel) and LLO25 were used for 3 h at room temperature, diluted 1:500 (CK 8.12) or 1:100 (LLO25) with 1% gelatine/0.01% NaN_3. After two washings with Tris-buffered saline with 0.05% Tween 20/L a 1:1000 diluted peroxidase conjugated goat anti-mouse IgG (H + L) antibody (Biorad, Richmond, CA) was used for 1 h as secondary antibody. After two further washings with Tris-buffered saline with 0.05%

[1]Marekov LN, Steinert PM: Evidence that the lipids of the epidermal cornified cell envelope are attached directly to involucrin. *J Invest Dermatol* 106:828A, 1996 (abstr.)

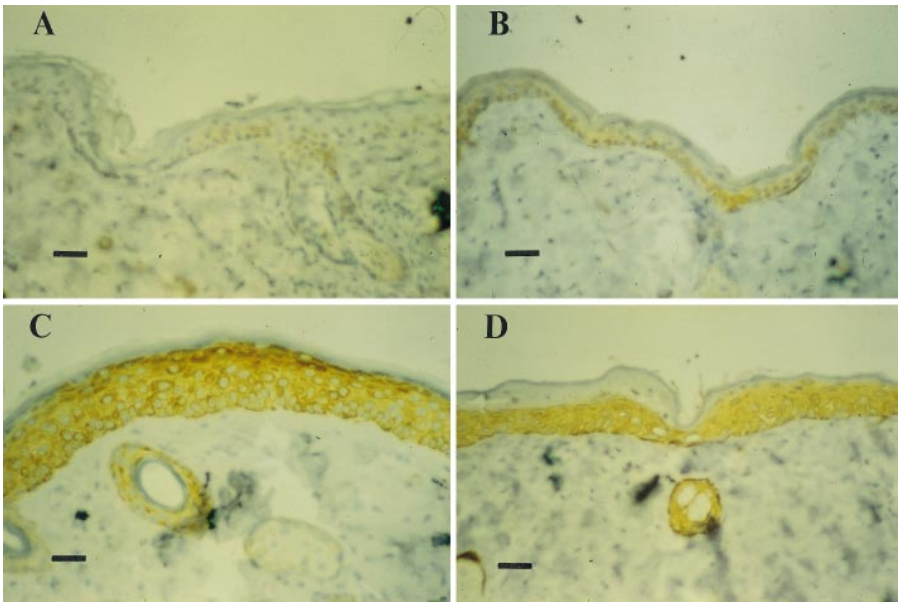

Figure 1. Immunostaining of keratin K6 after acetone-induced barrier disruption. (*A*) Normal mouse skin; (*B*) 12 h after barrier disruption; (*C*) 24 h after barrier disruption; (*D*) 24 h after barrier disruption + occlusion. Biopsies were sectioned and stained with K6 specific antibody. *Scale bar*: 15 μm.

Tween 20/L the bands were developed by addition of HRP-color development solution (4-chloro-1-naphthol/methanol/Tris-buffered saline/hydrogen peroxide). (ii) A horizontal semidry electroblotter with 0.2 μm nitrocellulose membrane, GB002 blotting paper (both from Schleicher & Schuell, Germany) was used with 0.048 M Tris, 0.039 M glycine, 20% methanol (pH 9.2) as transfer buffer. The blotting time was 2 h at 0.2 mA per cm^2, 100 V. Blocking was achieved with a mixture of 5% skim milk and 2% Tween 20 for ≈10 min. As primary antibody, 1:2000 diluted CK 8.12 (Sigma, St. Louis, MO) was used overnight and washed with 0.05 M Tris-Hcl, 0.5 M NaCl, 0.1% vol/vol Tween-20 (pH 9.0). As secondary antibody a rabbit anti-mouse peroxidase linked antibody (Sigma, 1:1000) was used for 3 h. For staining, 0.02% diaminobenzidine in a citrate/phosphate-buffer [0.035 M citric acid, 30% H_2O_2, 0.067 M Na_2HPO_4 (pH 5.0)] was used.

RESULTS

Permeability barrier disruption influences hyperproliferation-associated keratins K6, K16, and K17 In untreated hairless mouse epidermis as well as 3 h and 6 h after acetone-induced barrier disruption, we found no changes in the keratin expression either by immunostaining or by one- and two-dimensional gel electrophoresis. Keratin K6, K16, and K17 expression was first detected at 12 h after acute barrier disruption. The K6 antibody showed staining of basal and lower spinous layers (**Fig 1**). By two-dimensional gel electrophoresis we found small spots for keratins K16 and K17 at this time point (data not shown). Twenty-four hours after barrier disruption K6 immunostaining was noted in all nucleated epidermal layers (**Fig 1**), K16 was detected by one-dimensional gel electrophoresis (**Fig 2**, **Table I**), and K6, K16, and K17 were identified by two-dimensional gel electrophoresis. K6 was most visible by nonequilibrium pH gradient electrophoresis. K16 and K17 were clearly visible by equilibrium isoelectric focusing (**Fig 3**). Distinct expression for K6, K16, and K17 by immunostaining and by two-dimensional gel electrophoresis was also noted at 48 h and 72 h after barrier disruption.

In addition to acetone treatment we performed permeability barrier disruption by treatments with a detergent (SDS) and by tape-stripping to show that changes are not dependent on method, but are common to a variety of unrelated forms of disruption. After SDS or tape-stripping induced barrier disruption we also found the expression of keratins K6, K16, and K17 (examined only at 24 h after treatment) (data not shown). Occlusion for 24 h after acetone-induced barrier disruption led to a moderate focal reduction in K6 immunostaining (**Fig 1**) and to a slight decrease in K16 expression by one- and two-dimensional gel electrophoresis (**Fig 2**, **Table I**).

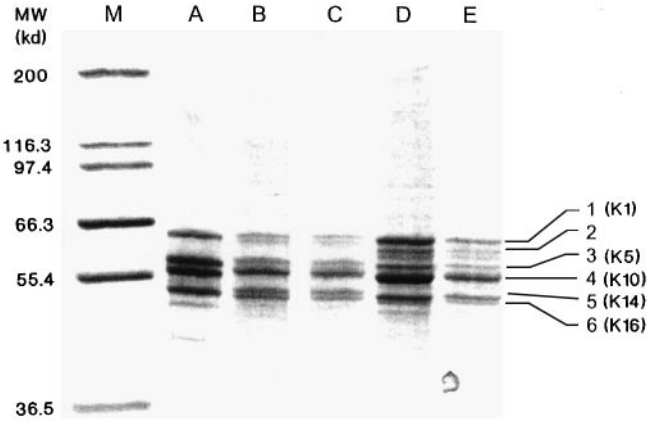

Figure 2. Keratins K1, K5, and K16 are influenced by acetone treatment ± occlusion and by EFAD ± occlusion (one-dimensional electrophoresis). *Lane a*, normal mouse skin; *lane b*, 24 h after acetone treatment; *lane c*, 24 h after acetone treatment + occlusion; *lane d*, EFAD (12th week); *lane e*, EFAD + occlusion (for 3 d). Keratins were purified by a standard cytoskeletal method and analyzed by SDS-PAGE.

The pattern of hyperproliferation-associated keratins in EFAD mice was similar to acetone-induced barrier disruption, and keratins K6, K16, and K17 were expressed (**Figs 2**, **3**, **Table I**). These changes in keratin staining pattern first occurred 6 wk after commencement of feeding an EFAD diet and increased while on a diet in weeks 9–12, in parallel with the ongoing defect of barrier function (TEWL: untreated, 8.7 ± 0.2 g per h per m^2; 9th week, 41.9 ± 9.2* g per h per m^2; 12th week, 67.3 ± 8.7* g per h per m^2; *$p < 0.01$, n = 3–9). In EFAD replete mice, after changing to a normal diet, TEWL and keratin pattern normalized within 2 wk.[2] Occlusion for 3 d in EFAD mice (12 wk on diet) led to a further increase in TEWL (Proksch *et al*, 1992), in contrast epidermal proliferation was reduced. BrdU labeling was 5-fold increased in EFAD (38.3 ± 6.15 $BrdU^+$-keratinocytes per mm epidermal basal membrane, 12th week) compared with normal

[2]Schmook FP, Pecanka R, Kowalsky E, Meingassner JG: Diet-induced changes in epidermal keratins in mice. *J Invest Dermatol* 105:495A, 1995 (abstr.)

Table I. Band intensities (peak areas) of keratins after acute or chronic barrier disruption ± occlusion (one-dimensional electrophoresis, Fig 2)[a]

Band (keratin)	Lane A (untreated) %	Lane B (acetone) %	Lane C (acetone + occlusion) %	Lane D (EFAD) %	Lane E (EFAD + occlusion) %
1 (K1)	16.71	6.97	9.12	23.45	18.15
3 (K5)	27.84	15.71	14.59	15.46	12.95
4 (K10)	30.40	37.45	38.37	35.85	39.93
5 (K14)	25.05	20.81	20.87	18.12	20.02
6 (K16)	0	19.07	17.05	7.12	8.94

[a]Dried gels were scanned, image analyzed (not shown), and calculated (integrated) by a ImageMaster 1D Elite System (band intensities in percentage of the total intensity of keratin peaks).

(8.01 ± 3.83, $p < 0.01$, $n = 5$). Occlusion in EFAD mice led to a 64% reduction in epidermal proliferation compared with unoccluded mice; however, this was still above normal (13.6 ± 4.84, $p < 0.025$, $n = 5$). Keratin K6, K16, and K17 expression was not significantly reduced by occlusion in EFAD (**Fig 2**, **Table I**).

Permeability barrier disruption influences basal (K5 and K14) and suprabasal (K1 and K10) keratins One-dimensional gel electrophoresis of keratins from the epidermis of untreated hairless mice showed four prominent keratin bands (1, K1; 3, K5; 4, K10; 5, K14). Twenty-four hours after experimental permeability barrier disruption by acetone the expression of the basal keratins K5 and K14 as well as expression of the suprabasal keratin K1 was reduced compared with normal. In contrast, the relative amount of suprabasal keratin K10 was increased. After chronic barrier disruption by EFAD, the expression to basal keratins K5 and K14 was reduced. In contrast, expression of suprabasal keratins K1 and K10 was increased compared with untreated epidermis. Occlusion after acute and chronic barrier disruption did not significantly influence the expression of basal or suprabasal keratins (**Fig 2**, **Table I**).

Permeability barrier disruption did not influence the expression of the cornified envelope protein loricrin in hairless mouse skin Loricrin immunostaining in untreated mouse skin was confined to the granular layers. Twelve, 24, and 48 h after barrier disruption, loricrin immunostaining was unchanged (data not shown). Neither barrier disruption by acetone, nor barrier disruption by SDS or by tape-stripping influenced loricrin expression in mouse skin. (We were unable to examine involucrin expression in mice, because there is no antibody available that works in skin sections of the mouse system.)

Permeability barrier disruption influences the expression of the cornified envelope protein involucrin but not loricrin in human skin In human skin, as in mouse skin, loricrin immunostaining was confined to the granular layers and did not change after experimental permeability barrier disruption (data not shown). Involucrin staining in untreated human skin as well as 6 h and 12 h after barrier disruption was confined to the granular and uppermost spinous layers. Changes in involucrin expression occurred 24 h and 48 h after treatment; staining was more pronounced and was extended to the middle spinous layers. After occlusion for 24 h there was a further extension of involucrin staining to the lower spinous layers (**Fig 4**).

DISCUSSION

We have shown that acute as well as chronic disruption of the epidermal permeability barrier results in changes in major structural proteins synthesized in the keratinocyte and related to proliferation and differentiation. Expression of the hyperproliferation-associated keratins K6, K16, and K17 determined by three different methods, was induced by acute barrier disruption. Similar expression of keratins has been described in human diseases that are accompanied by a disturbed permeability barrier. K16 is expressed in allergic and irritant contact dermatitis (acute or chronic disease) (Le *et al*, 1995) and K17 is expressed in a delayed type hypersensitivity induced by a mumps vaccine in human skin (Jiang *et al*, 1993). Also, K6, K16, and variable amounts of K17 are expressed in the hyperproliferative epidermis of psoriasis (a chronic disease) (Thewes *et al*, 1991; Moll, 1993; Smack *et al*, 1994); however, expression of these keratins is not necessarily linked to hyperproliferation. Kopan and Fuchs showed that the reduction in K6/K16 in retinoid-treated SCC-13 cultures was not accompanied by a decrease in cell proliferation (Kopan and Fuchs, 1989). In our study we were also interested in the time curve of the induction of keratins after acute permeability barrier disruption. K6, K16, and K17 were expressed as early as 12 h after acetone treatment and preceded epidermal hyperproliferation [we previously showed that an increase in DNA synthesis occurs 18–20 h after barrier disruption (Proksch *et al*, 1991)]. This also shows that the expression of these keratins is not a consequence of epidermal hyperproliferation. A similar time curve for the expression of K6 and K16 in healing of wounded human skin has been found previously and correlates with the onset of re-epithelization and a reorganization of keratin filaments in normal edge keratinocytes (Mansbridge and Knapp, 1987; Paladini *et al*, 1996). This reorganization of keratin filaments probably also occurs after permeability barrier disruption.

The relationship between permeability barrier function and keratin expression is also shown by our chronic EFAD model. In EFAD, barrier disruption occurs as a result of a metabolic disturbance. In these mice the lipid organization in the stratum corneum bilayers is disturbed because linoleic acid is replaced by oleic acid in ceramide 1 (Wertz *et al*, 1987; Hou *et al*, 1991). EFAD mice showed an increase in TEWL, but also an increase in lipid and DNA synthesis (Proksch *et al*, 1992). We then found that keratins K6, K16, and K17 are induced in EFAD. Staining of keratins changed with time during the EFAD diet, in parallel with the ongoing chronic permeability barrier disruption (increase in TEWL).

Occlusion after acetone treatment led to a slight decrease in the expression of the hyperproliferation-associated keratins K6 and K16, but did not influence K17. Previous studies have shown that the acetone-induced increase in lipid synthesis could be prevented by occlusion (Grubauer *et al*, 1987); however, in previous studies and in this study we also showed that the increase in epidermal proliferation induced by acute and chronic barrier disruption is only partially prevented by occlusion. DNA synthesis after acetone + occlusion or EFAD + occlusion is significantly reduced compared with unoccluded skin, but is still well above normal (Proksch *et al*, 1991). The small effect of occlusion on keratin expression after acute barrier disruption and the lack of effects after chronic barrier disruption also show that there is not necessarily a link between hyperproliferation and K6, K16, and K17 expression.

Basal and suprabasal keratins were also influenced by barrier disruption. In the acute model, expression of keratins K5 and K14 was decreased. Also, we noted a decrease in suprabasal keratin K1, contrasting with an increase in keratin K10. Decrease of the suprabasal differentiation-associated keratin K1 could be related to a disturbed differentiation after barrier disruption (Thewes *et al*, 1991). The small decrease in K14 was only noted because of the additional expression of K16. In EFAD mice, basal keratins K1 and K5 were reduced, and suprabasal keratins K1 and K10 were increased. This correlates with the increased thickness of the epidermis in EFAD (increase in the suprabasal compartment). The increase in suprabasal differentiation-

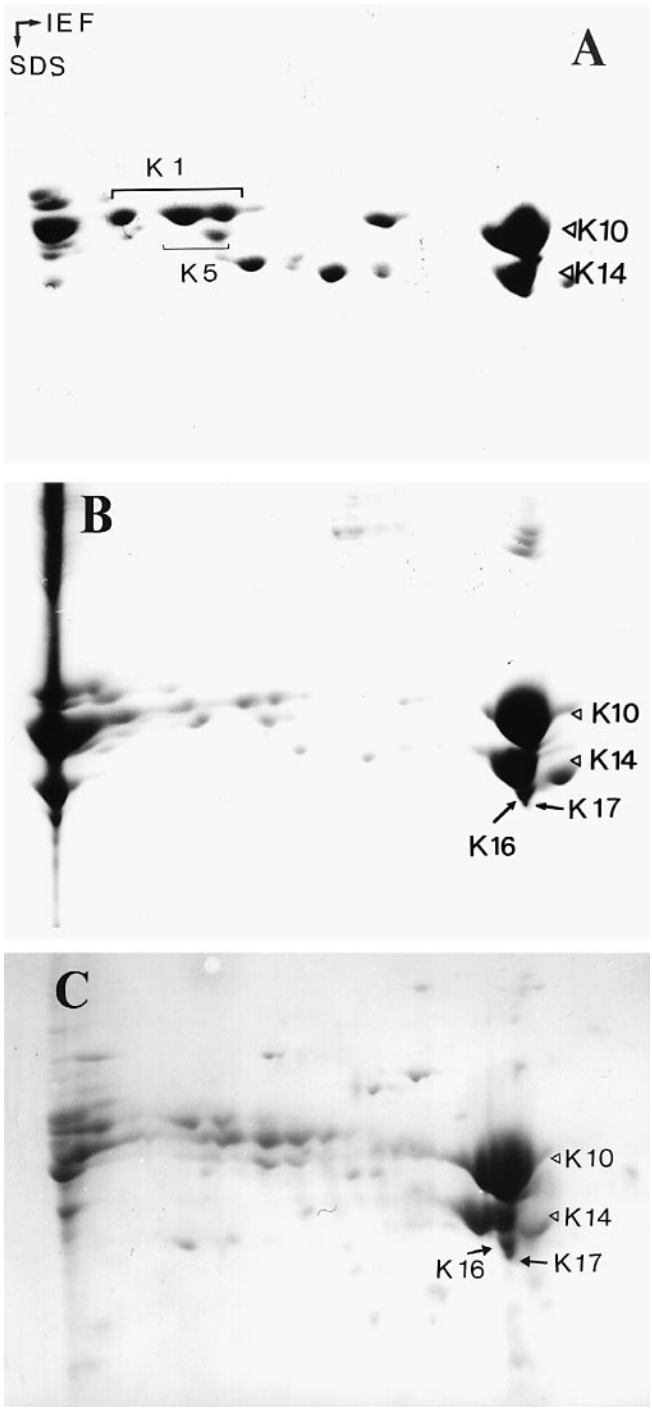

Figure 3. Keratins K16 and K17 are expressed after acetone- and EFAD-induced barrier disruption. (*A*) Normal mouse skin; (*B*) 24 h after acetone induced barrier disruption; and (*C*) EFAD mice. Keratins were extracted by a standard cytoskeletal method, separated initially by isoelectric focusing (pH 4–8), and then in the second dimension by SDS-PAGE.

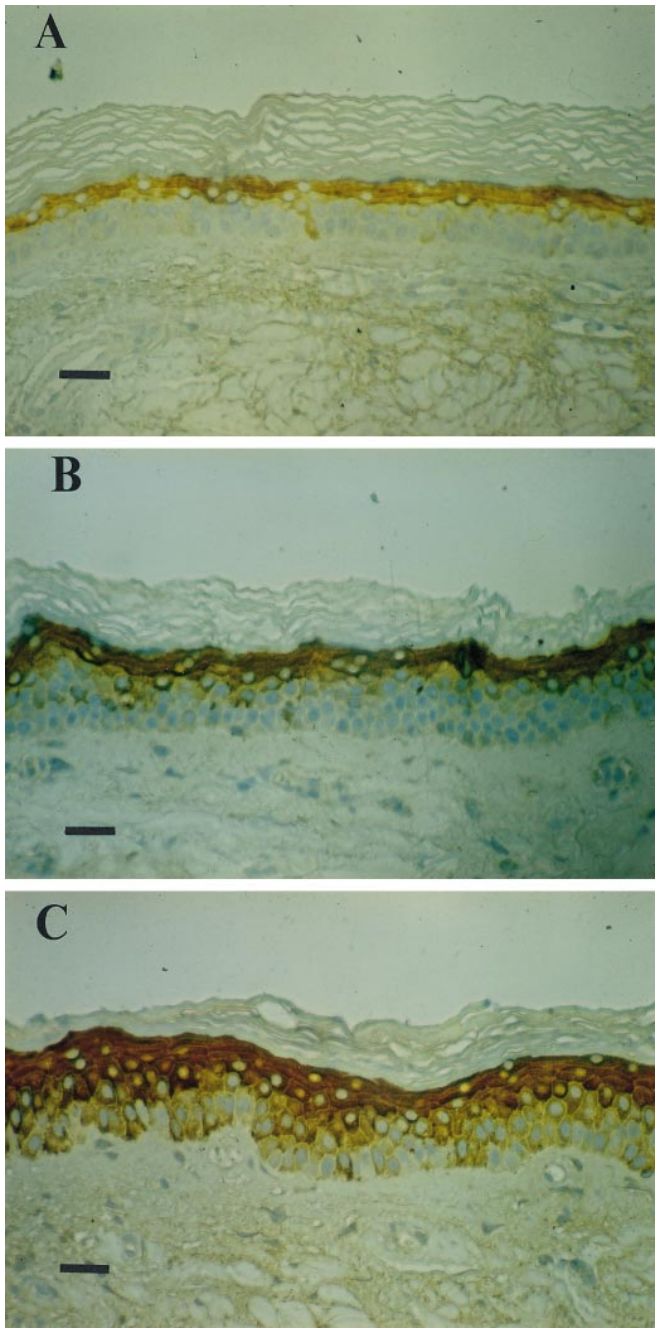

Figure 4. Premature immunostaining of involucrin after permeability barrier disruption. (*A*) Normal human skin; (*B*) 24 h after acetone-induced barrier disruption; (*C*) 24 h after barrier disruption + occlusion. Biopsies were sectioned and stained with an involucrin specific antibody. *Scale bar*: 15 μm.

associated keratins could be an attempt to repair the permeability barrier despite ongoing EFAD.

Surprisingly, different effects of barrier disruption on the expression of the two cornified envelope proteins involucrin and loricrin after barrier disruption was noted. Twenty-four hours after barrier disruption in human skin we found an extension of the involucrin expression from the granular layers and the upper spinous to the lower spinous layers (antibodies suitable for the mouse system were not available). This is in accordance with the results in diseased skin accompanied by a disturbed epidermal permeability barrier. In eczematous skin as well as in psoriasis an extension of involucrin expression to lower spinous layers has been reported (Hohl, 1990; Rosenthal *et al*, 1992; McKay *et al*, 1995; Proksch and Hagemann, 1996). Involucrin is a cytoplasmatic protein expressed early in keratinocyte differentiation and an early component of the cornified envelope (Robinson *et al*, 1996; Steinert *et al*, 1997). Loricrin expression, at the time points examined (12 h to 48 h after treatment), was unchanged after barrier disruption in mouse skin as well as in human skin. In eczema and psoriasis an unchanged or slightly reduced staining of loricrin has been noted (Hohl, 1993). Loricrin is expressed very late in epidermal differentiation and becomes cross-linked as a major component of the cornified envelope (Hohl, 1990; Steinert *et al*, 1995). It has previously been reported from cell culture studies involving retinoids that involucrin and loricrin synthesis are regulated by different mechanisms (Hohl *et al*, 1993).

Occlusion after acute barrier disruption in human skin did not reduce the expression of involucrin. As mentioned earlier, it was shown in mouse skin that occlusion reduced an acetone-induced increase in lipid synthesis and the increase in its rate-limiting enzymes towards normal (Grubauer *et al*, 1987; Proksch *et al*, 1990; Holleran *et al*, 1991; Ottey *et al*, 1995). For the determination of the lipid synthesis, occlusion after barrier disruption was performed for only 2–4 h, because the increase occurs rapidly. For involucrin expression we occluded for 24 h, because we found changes in involucrin expression without occlusion at that time point. It is difficult to obtain tightly fitting occlusion in human skin for such a long time period. Also, the effect of occlusion on epidermal proliferation is less pronounced in human than in mouse skin (Proksch *et al*, 1996). After irritation in human skin (SDS patch test application for 24 h, which also leads to barrier disruption) a reduction in epidermal proliferation by occlusion was not found at all (Welzel *et al*, 1996).

A link between keratins and barrier function is known in the human disease epidermolytic hyperkeratosis (also known as bullous congenital ichthyosiform erythroderma Broque), in which mutations of either keratin K1 or K10 have been identified as the underlying defect (Cheng *et al*, 1992; Chipev *et al*, 1992; Rothnagel *et al*, 1992). The epidermal permeability barrier is disturbed in this disease as has been shown previously by an increased TEWL (Frost *et al*, 1968). Interactions between keratins, cornified envelope proteins, and covalently bound lipids has been described by several authors (Hohl, 1990; Downing, 1992; Bickenbach *et al*, 1995; Steinert *et al*, 1995). Also, there is evidence that keratins are linked to the cornified envelope protein involucrin (Steinert *et al*, 1997). We now suggest that these interactions are influenced by skin barrier function.

Our findings clearly document that experimental permeability barrier disruption in acute and chronic models influences keratin polypeptides and the cornified envelope protein involucrin. The coordinated regulation of lipid synthesis, epidermal proliferation, and epidermal differentiation is critical for skin barrier function.

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REFERENCES

Bavandi A, Meingassner JG, Becker S: Diet-induced dermatitis response of hairless rats to systemic treatment with cyclosporin A (Sandimmun^{R}), cyclosporin H and FK506. *Exp Dermatol* 1:199–205, 1992

Bickenbach JR, Greer JM, Bundman DS, Rothnagel JA, Roop DK: Loricrin expression is coordinated with other epidermal proteins and the appearance of lipid lamellar granules in development. *J Invest Dermatol* 104:405–410, 1995

Cheng J, Syder AJ, Yu QC, Letai A, Paller AS, Fuchs E: The genetic basis of epidermolytic hyperkeratosis: a disorder of differentiation-specific epidermal keratin genes. *Cell* 70:811–819, 1992

Chipev CC, Korge BP, Markova N, Bale SJ, DiGiovanna JJ, Compton JG, Steinert PM: A leucine-proline mutation in the H1 subdomain of keratin 1 causes epidermolytic hyperkeratosis. *Cell* 70:821–828, 1992

Choi JS, Jackson SM, Elias PM, Feingold KR: The role of protein synthesis in permeability barrier homeostasis. In: Ohkawa A, McGuire J (eds). *The Biology of the Epidermis*. Amsterdam: Elsivier Science, 1992, pp. 11–19

Downing DT: Lipid and protein structures in the permeability barrier of mammalian epidermis. *J Lipid Res* 33:301–313, 1992

Elias PM: Epidermal lipids, barrier function, and desquamation. *J Invest Dermatol* 80:44–49, 1983

Frost P, Weinstein GD, Bothwell JW, Wildnauer R: Ichthyosiform dermatoses: III. studies of transepidermal water loss. *Arch Dermatol* 98:230–233, 1968

Fuchs E: Keratins and the skin. *Annu Rev Cell Dev Biol* 11:123–155, 1995

Grubauer G, Feingold KR, Elias PM: The relationship of epidermal lipogenesis. *J Lipid Res* 28:746–752, 1987

Grubauer G, Elias PM, Feingold KR: Transepidermal water loss: the signal for recovery of barrier structure and function. *J Lipid Res* 30:323–333, 1989

Hagemann I, Proksch E: Topical treatment by urea reduces epidermal hyperproliferation and induces differentiation in psoriasis. *Acta Derm Venereol (Stockh)* 76:353–6, 1996

Hazelbag HM, van der Broek LJCM, van Dorst EBL, Offerhaus GJA, Fleuren GJ, Hogendoorn PCW: Immunostaining of chain-specific keratins on formalin-fixed, paraffin-embedded tissues: A comparison of various antigen retrieval systems using microwave heating and proteolytic pre-treatments. *J Histochem Cytochem* 43:429–437, 1995

Hohl D: Cornified cell envelope. *Dermatologica* 180–201, 1990

Hohl D: Expression patterns of loricrin in dermatological disorders. *Am J Dermatopath* 15:20–27, 1993

Hohl D, Olano RB, de Viragh PA, Huber M, Detrisac CJ, Schnyder UW, Roop DR: Expression patterns of loricrin in various species and tissues. *Differentiation* 54:25–34, 1993

Holleran WM, Feingold KR, Mao-Qiang M, Gao WN, Lee JM, Elias PM: Regulation of epidermal sphingolipid synthesis by permeability barrier function. *J Lipid Res* 32:1151–1158, 1991

Hou SYE, Mitra AK, White SH, Menon GK, Ghadially R, Elias PM: Membrane structures in normal and essential fatty acid-deficient stratum corneum: characterization by ruthenium tetroxide staining and x-ray diffraction. *J Invest Dermatol* 96:215–223, 1991

Jackson SM, Word LC, Luner S, Taylor JM, Cooper AD, Elias PM, Feingold KR: Effect of cutaneous permeability barrier disruption on HMG-CoA reductase, LDL receptor, and apolipoprotein E mRNA levels in the epidermis of hairless mice. *J Lipid Res* 33:1307–1314, 1992

Jiang CK, Magnaldo T, Ohtsuki M, Freedberg IM, Bernerd F, Blumenberg M: Epidermal growth factor and transforming growth factor α specifically induce the activation- and hyperproliferation-associated keratins 6 and 16. *Proc Natl Acad Sci USA* 90:6786–6790, 1993

Kanitakis J, Zambruno G, Viac J, Thivolet J: Involucrin expression in keratinization disorders of the skin – a preliminary study. *Br J Dermatol* 117:479–480, 1987

Kopan R, Fuchs E: The use of retinoic acid to probe the relation between hyperproliferation – associated keratins and cell proliferation in normal and malignant epidermal cells. *J Cell Biol* 109:295–307, 1989

Laemmli UK: Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* 227:680–685, 1970

Lane EB, Rugg EL, Navasaria H, Leigh IM, Heagerty AHM, Ishida-Yamamoto A, Eady RAJ: A mutation in the conserved helix termination peptide of Keratin 5 in hereditary skin blistering. *Nature* 356:244–246, 1992

Le TKM, van der Valk PGM, Schalkwijk J, van de Kerkhof PCM: Changes in epidermal proliferation and differentiation in allergic and irritant contact dermatitis reactions. *Br J Dermatol* 133:236–240, 1995

Leigh IM, Navsaria H, Purkis PE, McKay IA, Bowden PE, Riddle PN: Keratins (K16 and K17) as markers of keratinocyte hyperproliferation in psoriasis *in vivo* and *in vitro*. *Br J Dermatol* 133:501–511, 1995

Mansbridge JN, Knapp AM: Changes in keratinocyte maturation during wound healing. *J Invest Dermatol* 89:253–263, 1987

McKay IA, Leigh IM: Altered keratinocyte growth and differentiation in psoriasis. *Clinics Dermatol* 13:105–114, 1995

Menon GK, Feingold KR, Elias PM: Lamellar body secretory response to barrier disruption. *J Invest Dermatol* 98:279–289, 1992

Ming ME, Daryanani HA, Roberts LP, Baden HP, Kvedar JC: Binding of keratin intermediate filaments (K10) to the cornified envelope in mouse epidermis: implications for barrier function. *J Invest Dermatol* 103:780–784, 1994

Moll I: Cytokeratine. *Hautarzt* 44:491–500, 1993

O'Farell PZ, Goodman HM, O'Farell PH: High resolution two-dimensional electrophoresis of basic as wells as acid proteins. *Cell* 12:1133–1142, 1977

Ottey KA, Wood LC, Grunfeld C, Elias PM, Feingold KR: Cutaneous permeability barrier disruption increases fatty acid synthetic enzyme activity in the epidermis of hairless mice. *J Invest Dermatol* 104:401–404, 1995

Paladini RD, Takahashi K, Bravo NS, Coulombe PA: Onset of reepithelization after skin injury correlates with a reorganization of keratin filaments in wound edge keratinocytes: defining a potential role for keratin 16. *J Cell Biol* 381–397, 1996

Proksch E, Hagemann I: Topical treatment by urea reduces epidermal hyperproliferation and induces differentiation in psoriasis. *Acta Derm Venereol (Stockh)* 76:353–356, 1996

Proksch E, Elias PM, Feingold KR: Regulation of 3-hydroxy-3-methylglutaryl- coenzyme a reductase activity in murine epidermis. *J Clin Invest* 85:874–882, 1990

Proksch E, Feingold KR, Elias PM: Barrier function regulates epidermal DNA- synthesis. *J Clin Invest* 87:1668–1673, 1991

Proksch E, Feingold KR, Elias PM: Epidermal HMG CoA reductase activity in essential fatty acid deficiency: barrier requirements rather than eicosanoid generation regulate cholesterol synthesis. *J Invest Dermatol* 99:216–220, 1992

Proksch E, Brasch J, Sterry W: Integrity of the permeability barrier regulates epidermal Langerhans cell density. *Br J Dermatol* 134:630–638, 1996

Rice RH, Green H: Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* 18:681–694, 1979

Robinson NA, Latell PT, Eckert RL: Involucrin is a covalently cross-linked consistent of highly purified epidermal corneocytes: evidence for a common pattern of involucrin cross-linked *in vivo* and *in vitro*. *J Invest Dermatol* 107:101–107, 1996

Roop D: Defects in the barrier. *Science* 267:474–476, 1995

Rosenthal DS, Griffiths CEM, Yuspa SH, Roop DR, Voorhees JJ: Acute or chronic topical retinoic acid treatment of human skin in vivo alters the expression of epidermal transglutaminase, loricrin, involucrin, filaggrin and keratins 6 and 13 but not keratins 1, 10, and 14. *J Invest Dermatol* 98:343–349, 1992

Rothnagel JA, Dominey AM, Dempsey LD, *et al*: Mutations in the rod domains of keratins 1 and 10 in epidermolytic hyperkeratosis. *Science* 257:1128–1130, 1992

Schweizer J: Murine epidermal keratins. In: Darmon M, Blumenberg M (eds). *Molecular Biology of the Skin*: *The Keratinocyte*. San Diego: Academic Press, 1993, pp. 33–78

Smack PD, Korge BP, James WD: Keratin and keratinization. *J Am Acad Dermatol* 30:85–102, 1994

Steinert PM, Marekov LN: The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross- linked

components of the human epidermal cornified cell envelope. *J Biol Chem* 270:17702–17711, 1995

Steinert PM, Marekov LN: Direct evidence that involucrin is a major early isopeptide cross-linked of the keratinocyte cornified cell envelope. *J Biol Chem* 272:2021–2030, 1997

Thewes M, Stadler R, Korge B, Mischke D: Normal psoriatic epidermis expression of hyperproliferation-associated keratins. *Arch Dermatol Res* 283:465–471, 1991

Welzel J, Wilhelm KP, Wolff HH: Skin permeability barrier and occlusion: no delay of repair in irritated human skin. *Contact Dermatitis* 35:163–168, 1996

Wertz PW, Swartzendruber DC, Abraham WH, Madison KL, Downing DT: Essential fatty acids and epidermal integrity. *Arch Dermatol* 123:1381–1384, 1987

Wertz PW, Madison KC, Downing DT: Covalently bound lipids of human stratum corneum. *J Invest Dermatol* 92:109–111, 1989

Wild G, Mischke D: Variation and frequency of cytokeratine polypeptide patterns in human squamous non-keratinizing epithelium. *Exp Cell Res* 102:114–126, 1986

Wilson AK, Coulombe PA, Fuchs E: The roles of K5 and K14 head tail and R/KLLEGE domains in keratin filament assembly in vitro. *J Cell Biol* 119:401–414, 1992

Yoshikawa K, Katagata Y, Kondo S: Relative amounts of keratin 17 are higher than those of keratin 16 in hair-follicle-derived tumors in comparison with nonfollicular epithelial skin tumors. *J Invest Dermatol* 104:396–400, 1995

Zouboulis CC, Korge BP, Mischke D, Orfanos CE: Altered proliferation, synthetic activity, and differentiation of cultured human sebocytes in the absence of vitamin A and their modulation by synthetic retinoids. *J Invest Dermatol* 101:628–633, 1993